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Identification of vitronectin as a novel insulin-like growth factor-II binding protein

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Abstract. We have previously reported the presence of a 70 kDa insulin-like growth factor (IGF)-II-specific binding protein in chicken serum using Western ligand blotting approaches. In order to ascertain the identity of this 70 kDa IGF-II binding species, the protein has been purified from chicken serum using a combination of ion-exchange and gel-permeation chromatography. Interestingly, amino acid sequencing of the purified protein revealed that it has the same N-terminal sequence as chicken vitronectin (VN). The protein has the ability to specifically bind IGF-II and not IGF-I as determined by ligand blotting, cross-linking and competitive binding assay approaches. In addition, the protein binds ^{125}I-des(1-6)-IGF-II, suggesting that the interaction with IGF-II is different to those with other characterized IGF-binding proteins. Importantly, we have ascertained that both human and bovine VN also specifically bind IGF-II. These results are particularly relevant in the light of the recent report that the urokinase-type plasminogen activator receptor, a protein that also binds VN, has been shown to associate with the cation-independent mannose-6-phosphate/IGF-II receptor and suggest a possible role for IGF-II in cell adhesion and invasion.

We, along with others, have reported the presence of a 70 kDa insulin-like growth factor (IGF)-II-specific binding protein in chicken serum using Western ligand blotting approaches (1-4). Furthermore, *in vivo* studies examining the association of radiolabeled human and chicken IGF-II with IGF-binding proteins (IGFBPs) in the circulation of chickens suggest that the 70 kDa protein is a major carrier of IGF-II in chicken serum (3). This finding is intriguing given that the chicken cation-independent mannose-6-phosphate receptor (CI-MPR), unlike its mammalian homologues, does not bind IGF-II (5). Hence we speculated that this protein may be the functional equivalent of the mammalian circulating CI-MPR or a novel IGF-II regulatory protein (1).

More recently we have found that receptor binding studies performed in monolayers of cultured cells grown in the presence of fetal bovine serum (FBS) and using radiolabeled IGF-II, but not IGF-I, produce anomalous results due to unexpected binding of radiolabeled IGF-II to the culture plates. Accordingly, the nature of this apparently specific IGF-II binding has been investigated. In this study we report that chemical cross-linking of chicken serum and FBS proteins following adsorption to culture plates reveals that a 70 kDa protein is responsible for the receptor assay interference. Moreover, we have purified the 70 kDa protein from chicken serum and N-terminal protein sequencing reveals that the novel IGF-II specific binding protein is vitronectin (VN).

Methods

Proteins: Human and chicken IGFs and the des(1-6)-IGF-II variant were from GroPep Pty Ltd, Adelaide, Australia, while insulin was purchased from Novo Nordisk Pharmaceuticals Pty Ltd, North Ryde, Australia. Human and bovine VN were from Promega Corporation, Madison, WI, and Sigma Chemical Co, St Louis, MO, respectively. Proteins were iodinated to specific activities between 30 and 60 μCi/μg with chloramine-T.

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Cross-linking studies: Twenty-four-well tissue culture plates were exposed to 1 ml of DMEM containing 10% (v/v) chicken serum or FBS for 24 hr prior to washing twice with 2 ml binding buffer and performing binding and cross-linking experiments as described by Ballard *et al.* (6).

Purification of the 70 kDa IGF-II specific binding protein from chicken serum: Chicken serum from 6 week old broiler chickens (400 ml) was defatted with 1,1,2-trichloro-1,2,2-trifluoroethane prior to being loaded on to a Sepharose Fast Flow S (Pharmacia, North Ryde, Sydney, Australia) column (5 cm diameter x 5 cm) equilibrated with 50 mM tri-sodium citrate at pH 6. The column was re-equilibrated prior to elution of protein from the column with a gradient from 0-2 M NaCl in 50 mM tri-sodium citrate at pH 6, over 30 min at a flow rate of 4 ml/min. Fractions containing the 70 kDa IGF-II binding species, as detected by ligand blotting (7) using ^{125}I-labeled chicken IGF-II, were subjected to size-exclusion chromatography in 50 mM Na_2HPO_4/150 mM NaCl at pH 7.2 using a column (5 cm diameter x 100 cm) packed with Cellufine GCL 1000M (Amicon Corporation, Danvers, MA). Fractions identified as containing the 70 kDa IGF-II binding protein were then loaded on to a Sepharose Fast Flow Q (Pharmacia, North Ryde, Sydney, Australia) column (5 cm diameter x 5 cm) equilibrated with 50 mM tri-sodium citrate at pH 6. Following re-equilibration of the column after loading, the protein was eluted from the column with a gradient from 0-2 M NaCl in 50 mM tri-sodium citrate at pH 6, over 30 min at a flow rate of 2 ml/min. Fractions containing the 70 kDa IGF-II binding species were pooled, electrophoresed on a 10% SDS-polyacrylamide gel run under non-reducing conditions and the proteins transferred to PVDF membrane. Following staining of the PVDF membrane with Coomassie Blue, the band at 70 kDa, representing the major protein species present, was cut from the membrane and subjected to N-terminal amino acid sequencing.

Two-dimensional SDS-polyacrylamide gel analysis of proteins: Two dimensional (2-D) SDS-PAGE analysis was

performed using the Pharmacia Mighty Small Tube Gel Adapter Kit and the protocols suggested by the supplier. The first dimension separation utilised an isoelectric focussing tube gel incorporating pH 3-10 ampholines, while the second dimension separation was run on a 10% SDS-polyacrylamide gel. Following separation of the proteins in the two-dimensions, the proteins were transferred to nitrocellulose and probed with ^{125}I-labeled IGF-II using the ligand blotting procedure (7).

Binding Assays: Assays were performed in removable Immulon-4 wells (Dynex Technologies Inc, Chantilly, VA) pre-coated with human VN by incubating 100µl of VN diluted in HEPES-binding buffer for 5 hr at at 4 °C. Binding of ^{125}I-labeled human IGF-II to VN-coated wells was measured in the presence and absence of competing growth factors after 18 hr at 4 °C as described by Ballard *et al.* (8) with the following exceptions. Firstly, the final assay volume was 100 µl/well and secondly, following washes to remove unbound IGF-II, the radioactivity remaining in the removable wells was directly counted in a gamma counter.

Results

Crosslinking experiments followed by SDS-PAGE analysis were performed using either chicken or human radiolabeled IGF-II in binding studies with culture plates exposed to media containing chicken serum or FBS in the absence of cells. We found that one of the IGF-II binding proteins adsorbed to the culture plates in both the chicken serum and FBS studies has a molecular mass of approximately 70 kDa (Fig.1A). Moreover, binding of ^{125}I-labeled IGF-II to the 70 kDa species appeared to be specific because IGF-II (lanes 4 and 6), but not IGF-I (lane 7) nor insulin (lane 2) displaced the radiolabel bound to this protein. In addition, similar specific binding to the 70 kDa species was observed when the adsorbed proteins were crosslinked to ^{125}I-labeled des(1-6)-IGF-II (Fig. 1B), an IGF-II analog with poor binding to known IGFBPs (9), suggesting that the nature of this binding interaction may be different to that with other IGFBPs.

In order to characterize the novel 70 kDa IGF-II binding species, the protein was purified from chicken serum using a combination of ion-exchange and gel-permeation chromatography. The fractions from the purification were monitored by SDS-PAGE in conjunction with Western ligand blotting. N-terminal protein sequencing of the purified proteins following separation by SDS-PAGE and transfer to PVDF membrane indicated that the IGF-II-specific binding protein was approximately 80% pure. Moreover, the residues identified from 20 cycles of N-protein terminal sequencing, AEDS-EGR-DEGFNAMKK-Q, are identical to those reported for chicken VN. In addition, the minor sequences identified by amino acid sequencing were fragments of VN cleaved near the RGD cell recognition sequence flanking the N-terminal somatomedin B domain (10). The size and charge heterogeneity of the purified protein was also revealed by 2-dimensional SDS-PAGE. However, all proteins detected by Coomassie Blue staining were demonstrated to also bind radiolabeled IGF-II following transfer to nitrocellulose and Western ligand blotting (Fig.2). Thus, it is highly unlikely that the interaction of IGF-II with VN results from binding to a co-purified contaminant.

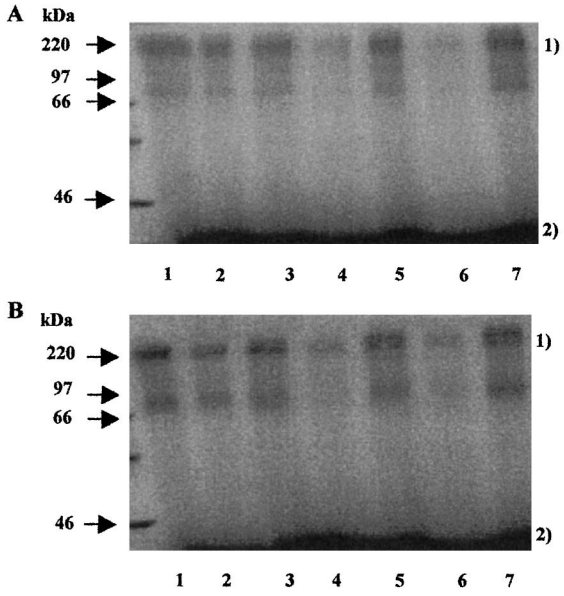

FIG.1 Chemical crosslinking of ^{125}I-labeled IGF-II to proteins in fetal bovine serum. SDS-PAGE of FBS proteins bound to tissue culture plates crosslinked to ^{125}I-labeled human IGF-II (A) or ^{125}I-labeled des(1-6)-human IGF-II (B). Crosslinking of proteins was performed in the presence of 10 ug insulin (lane 2), 20 ng human IGF-II (lane 3), 200 ng human IGF-II (lane 4), 20 ng chicken IGF-II (lane 5), 200 ng chicken IGF-II (lane 6) and 200 ng human IGF-I (lane 7). Molecular weight markers are indicated and lane 1 is a control where no treatments were added. IGF-II binding complexes with masses of approximately 220 (1) and 30 kDa (2) presumably represent IGF-II binding to the circulating form of the CI-MPR (1) and IGFBPs (2).

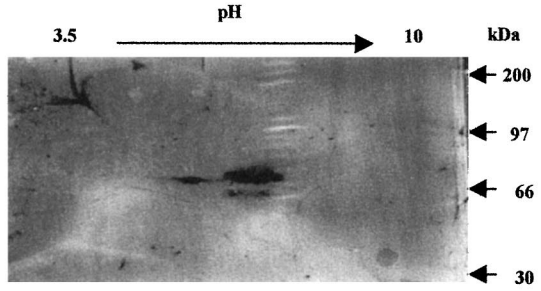

FIG.2 Ligand blotting analysis of the purified IGF-II-specific chicken binding protein. Ligand blotting of the purified protein with ^{125}I-labeled chicken IGF-II following 2-D SDS-PAGE and transfer to nitrocellulose.

The purified protein has the ability to specifically bind either human (Fig. 3, lane 3) or chicken (lane 5) radiolabeled IGF-II as determined by Western ligand blotting. Moreover, the binding is displaceable by IGF-II (lane 9) and not by IGF-I (lane 7), supporting our previous observations with ligand blotting of chicken serum (1) and the cross-linking data we report here. In addition, SDS-PAGE of commercially sourced human VN followed by ligand blotting with either radiolabeled human (lane 2) or chicken IGF-II (lane 4) indicates that mammalian VN can also bind IGF-II. Furthermore, N-terminal protein sequencing and 2-D SDS-PAGE has established that the human VN used in these studies is pure. Similar results were obtained with bovine VN (results not shown).

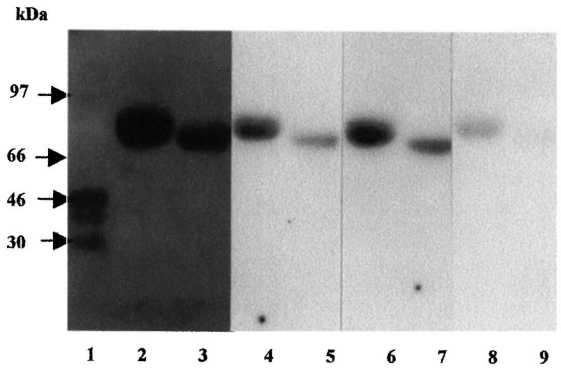

FIG.3 Western ligand blot analysis of radiolabeled IGF-II binding to chicken and human VN. Ligand blotting of FBS (1.8 µl, lane 1), human VN (10 µg, lanes 2, 4, 6, 8) and purified chicken VN (5 µg, lanes 3, 5, 7, 9) probed with ^{125}I-labeled human IGF-II (lanes 1-3), or ^{125}I-labeled chicken IGF-II (lanes 4-9), in the presence of unlabeled chicken IGF-I (lanes 6, 7), unlabeled chicken IGF-II (lanes 8, 9) or with no added growth factors (lanes 1-5).

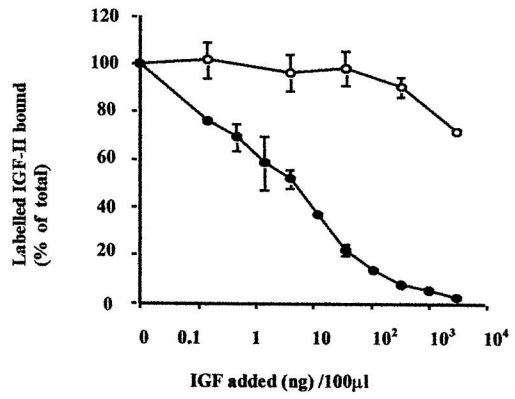

FIG.4 Binding of IGF-II to VN in solid-plate assays. Competition by human IGF-II (•) and human IGF-I (o) for binding of ^{125}I-labeled human IGF-II to 500 ng of human VN coated on Immulon-4 removable wells. Error bars indicate the S.E.M. at each concentration as calculated from a total of six determinations in two experiments.

Radioligand binding assays confirmed that ^{125}I-labeled IGF-II bound to VN in a concentration-dependent manner. Furthermore, half-maximal competition for binding of ^{125}I-labeled IGF-II to 500 ng of human VN was observed at a concentration of 4.32 ng/100 µl, while the K_d of the interaction was calculated to be 5.57 nM. In addition, IGF-I was much less effective in displacing the binding of IGF-II to VN, requiring approximately 5000-fold higher concentrations to achieve similar effects to those obtained with IGF-II (Fig. 4).

Discussion

We report here the purification and characterization of a novel IGF-II-specific binding protein from chicken serum. Amino acid sequencing has revealed that the purified protein has an identical amino acid sequence to that reported for chicken VN while the minor protein contaminants sequenced were actually IGF-II-binding fragments of chicken VN (10). Furthermore, the purified protein specifically binds IGF-II and not IGF-I, in accord with our cross-linking experiments (Fig. 1) and earlier findings with ligand blotting studies (1). Interestingly, the cross-linking studies revealed that the IGF-II analogue, des(1-6)-IGF-II, a protein with much reduced affinity for IGFBPs (9), retains the ability to bind to the 70 kDa protein. This suggests that the binding interaction between IGF-II and VN may involve residues in IGF-II other than those involved in binding to IGFBPs. Amino acid sequence analysis of chicken VN reveals minimal identity with IGFBPs, or indeed with IGFBP-related proteins (11), providing further evidence that the interaction of IGF-II with VN is different to that with classic IGFBPs. Moreover, the affinity of the interaction appears to lower than that reported for IGFBPs which bind the IGF ligands with K_d values of approximately 0.1 nM (11).

The demonstration that human and bovine VN can also specifically bind IGF-II gives further credence to this unexpected result with chicken VN. Indeed, the adhesive characteristics of VN, otherwise known as "serum-spreading factor", also explained some of the difficulties encountered in the purification of the protein. Curiously, VN has previously been described as a mitogenic factor termed "somatomedin B" (12). However, the reported biological activity of "somatomedin B" was later attributed to the presence of co-purified growth factor contaminants (13, 14). Our finding that IGF-II binds to VN now offers a plausible explanation for the enigmatic mitogenic activity that was ascribed to "somatomedin B".

While this paper represents the first report describing a specific binding interaction between VN and a growth factor, further studies are required to establish the functional importance of the interaction. Nevertheless, our previous investigations suggest that in chickens at least, the 70 kDa protein which we have now identified as VN, is potentially a major carrier of IGF-II in the circulation (3). Moreover, this role has been demonstrated to be unaffected by nutritional status (4). Indeed, the absence of IGF-II-binding forms of the CI-MPR in chickens may necessitate such a

physiological function for VN in aves (5). An uncharacterized IGF-II-specific binding protein of similar size has also been reported in sheep plasma, but whether this protein is VN remains to be determined (15).

While there is no evidence yet to support a functional role for IGF-II binding to VN in the extracellular matrix, recent reports describe similar interactions of growth factors with extracellular matrix proteins. For example, perlecan binds fibroblast growth factors (16) while decorin has been demonstrated to interact with the epidermal growth factor system (17). These extracellular matrix proteins act as "co-receptors", providing a reservoir of growth factors at the cell surface and perhaps a similar role is served by the VN:IGF-II interaction. Interestingly, IGF-II has recently been demonstrated to enhance invasion by extravillous trophoblast (EVT) cells by stimulating migration. This response was not observed upon addition of IGF-I, nor was the response mediated by the type-1 IGF receptor. However, IGF-II specific-binding proteins were identified on the EVT cell surface including a 75 kDa protein and a protein of the size expected for the CI-MPR. While the identity of the 75 kDa protein has not been established, it is possible, given its size and preference for binding IGF-II, that it is VN. Moreover, the invasion assays performed with these cells were undertaken in the presence of 1% FBS, hence VN, and the IGF-II-induced migration was drastically reduced in serum-free conditions (18). In addition, an unidentified 70 kDa IGF-II-specific binding protein has also been reported in an erythroleukemia cell line that proliferates in response to IGF-II in the absence of the type-1 IGF receptor (19). The recent finding that the urokinase-plasminogen activator receptor, a protein that binds VN, also binds to the CI-MPR or type-2 IGF receptor, adds another intriguing twist to the way by which VN may modulate IGF-II action (20). Indeed it will be interesting to determine whether components of the IGF, urokinase and CI-MPR systems interact to regulate IGF-II action at the cellular level, particularly with respect to IGF-II-induced migration/invasion.

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